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Dephosphorylation of Cdc20 is required for its C-box-dependent activation of the APC/C

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 15 February 2012

Thank you for submitting your manuscript on Cdc20 phosphorylation-regulation to The EMBO Journal. It has now been reviewed by three expert referees, and I am happy to inform you that all of the consider your findings potentially important and in principle suited for publication in our journal, pending adequate clarification of a number of major and minor concerns. In this respect, I feel the most important aspect that need to be addressed are referee 1's major points 1 and 2, as well as referee 2's first point. In addition, referee 3 also raises several important issues (especially points 1-4), including the request to show characterization of the used Cdc20 T79 phospho-specific antibody.

Should you be able to satisfactorily respond to these points in a revised version of the manuscript, then would should be able to consider the study further for publication. When revising the work, please make sure to also pay close attention to the various other (minor) points raised, including the requests for controls, molecular weight markers, proper statistical analysis, and various aspects of presentation including introductory statements. With regard to presentation, I would also kindly urge you to heed referee 3's concerns regarding the use of the terms 'Fizzy' and 'Fizzy-related', which I overall agree with (despite being myself well-aware and fond of these names and their history).

I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version. When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1

Cdc20 phosphorylation has been suggested to promote Cdc20 incorporation into the mitotic checkpoint complex (MCC) and thereby inhibit APC/C activity. Here, Labit et al. use Xenopus egg extracts to study spindle assembly checkpoint (SAC)-independent functions of Cdc20 phosphorylation. They identify three CDK consensus sites (T64, T68, T79) in the N terminus of Cdc20 whose phosphorylation antagonises APC/C binding and activation. They show that phosphorylation of all three sites prevents binding to the APC6 subunit of the APC/C whereas mutation of the adjacent C-Box impairs recruitment to APC8. They raise a phospho-specific antibody against phospho-T79 and confirm that T79 is primarily dephosphorylated in Fizzy/Cdc20 bound to active 'anaphase' APC/C. The authors present evidence that PP2A, and potentially other okadaic acid-sensitive phosphatases, contributes to Cdc20 de-phosphorylation and thereby to APC/C activation. Based these findings the Labit et al. propose a positive feedback loop in which the loss of CDK activity consequent to APC/C-dependent cyclin degradation acts in concert with Cdc20 de-phosphorylation to increase APC/C activity and drive exit from mitosis.
Overall this is an interesting manuscript in which the experiments are carefully performed and well controlled. It is intriguing that phosphorylation of the same CDK sites (T64, T68, T79) that have been proposed to be important for Cdc20 incorporation into the MCC also prevent APC/C binding. The results indicate that Cdc20 phosphorylation prevents activation of the APC/C by two independent mechanisms. However, there is a concern regarding the assay for Cdc20 with the 'anaphase' APC/C in that the authors predominantly use the N terminus of Cdc20 in their experiments, which only promotes ubiquitylation of Nek2a, and Nek2a is a prometaphase substrate of the APC/C. Hence, the following points should be addressed before publication:

Major Points:
1) I am concerned that the amino-terminus of Cdc20 may not recapitulate the behavior of the intact Cdc20 protein in all circumstances. The authors should replace the endogenous Cdc20 in cycling extracts (figure 5E) with a full-length Cdc20 phospho-mimetic (5E) or a full-length alanine mutant (5A) to compare the onset of Cyclin B and securin destruction. There is also a concern with Xenopus extracts as a model for mitosis since they are in essence a meiotic system. (It would greatly strengthen the paper if the authors showed that the de-phosphorylation of T64, T68, T79 is required for normal progression through mitosis by siRNA and rescue in human cells, for example.) In the absence of this the authors should include appropriate caveats in the text.
2) The authors show that the phosphorylated N terminus of Cdc20 cannot support the degradation of Nek2a (figures 1E, 4C). How can this result be reconciled with the SAC-independent degradation of Nek2a in prometaphase when CDK activity is high and T64, T68, T79 are likely to be fully phosphorylated?
3) The authors should quantify the assays shown in Figure 4B and 5A and present these as means +/- SDs.
4) The authors should modify the Introduction. They make the surprising statement that structural data on APC/C is limited and fail to cite the recent structural papers from the Barford lab (Schreiber et al., 2011; da Fonsec et al., 2011). They also state that Cdc20-APC/C degrades securin and Cyclin B in anaphase whereas the key phase for this is metaphase. (Note that anaphase, as defined by sister chromatid separation, cannot take place if securin is not degraded.) Lastly, they should mention that Cdh1 is primarily regulated by Emi1 in interphase in animal cells.

Minor Points:
a) The authors should indicate molecular mass markers on all Western blots in figures 1-6

b) Error bars should be added to the quantifications shown in figures 2B, 3A-C and 3F.
c) I don't think that the data in Fig 1E justify the statement that okadaic acid abolishes the activation by N159: reduces would be more appropriate.
c) The reference to Figure 6C needs to be added to the text (page 13)
d) The model (figure 7) should be more precise regarding the mitotic phases (i.e. prometa-, meta-, anaphase) in which Cdc20 phosphorylation and de-phosphorylation occurs.

Referee #2

General comments :
The paper entitled "Dephosphorylation of Fizzy/Cdc20 is required for its C-box-dependent activation of the APC/C" by H. Labit et al. reports new mechanism by which APC/C, an E3 ubiquitin ligase, is activated before CDK is inactivated. They found that the dephosphorylation of Fizzy in the presence of high CDK activity is important for APC/C activation. The observation in this manuscript accounts well for how CDK-dependent phosphorylations that of APC/C and Fizzy have opposite effects on E3 ligase activity is coordinated by opposing phosphatases. The 3 CDK sites of Fizzy were preferentially and continuously dephosphorylated by PP2A and some other unknown phosphatases even during mitosis. These results would give us new insights not only on APC/C regulation itself, but also on how the balance of kinase/phosphatase could varies. They further showed in the last part how the dephosphorylated state of Fizzy mechanistically contributes to the affinity of Fizzy to the body of APC/C. The data are in good quality, and the experiments were very well designed. But still I would like to suggest one experiment (#1) that would strengthen their claims and is doable with their reagents and technique. In addition, there are minor points (#2 to 11) to improve the clarity of this manuscript.

Specific points :
#1, To check how quickly the T79 phosphorylation of Fizzy is dephosphorylated in anaphase, the authors used anaphase egg extract supplemented with p27 (Figure 5C). But p27 forces the extract to exit from anaphase and to go into interphase (judging from the downshift of Apc3 shown in the top panel). So this setting does not directly reflect the turnover speed of T79 phosphorylation in anaphase. It would be better to see the speed of the loss of radioactivity from N159-T79 fragment (by using Cdk and [g-32P]-ATP). If this is compared with the S50, S114 or some other stably-phosphorylated substrate, it would be perfect.
#2, Figure 1B, 1C and S1A should have stained gel images, as the quantity of added proteins are important here.
#3, Page 7, lines 7-9. The description about Figure 1E is an exaggeration. The N159 fragment does support Nek2A degradation (lanes 9-16), if you compare it with lanes 1-4 of the same panel. Of course, I agree that OA and phosphorylation of N159 weaken (but not abolish) the impact of N159 on Nek2A degradation.
#4, Please describe in the main text why MG132 was added in the Figure 1A. Is this to avoid mitotic exit that could be induced by 5A protein?
#5, The position (mobility) of Apc3 in the right panel of Figure 2C looks intermediate, compared with that in the left panel. Is this phosphorylated (mitotic) form? Or is Apc3 partially dephosphorylated by the addition of 5A?
#6, Readers would need some explanation about the CSF state and differences between CSF (a kind of metaphase?) and anaphase extract.
#7, What kind of egg extract was used in the Figure 2C? Probably it is CSF, but not clearly described anywhere.
#8, The symbol (-p) for normal phosphorylation in the Figure 1D & 1E is same as one for thiophosphorylation in the Figure 4B & C. Or is the N159 protein used in the Figure 1E also thiophosphorylated? If they are different, please use different symbols for clarity.
#9, Please specify what IVT is, when it is first used (page 31, line 15).
#10, Please specify the delta-Cb mutation. Is this a substitution mutant or depletion?
#11, Boundaries of the short Fizzy fragments are not clear in the text or figures. For example, is 27th amino acid of Fizzy included in the fragment 1, 2 or both of them (Figure 1B)?

Referee #3

This paper describes studies of a regulatory mechanism that helps control the activation of Cdc20-APC/C at the beginning of anaphase. The authors present evidence to suggest that (1) Cdc20 is phosphorylated at multiple sites in its N-terminal region by Cdk, (2) phosphorylation of a subset of these sites inhibits Cdc20 function, and (3) phosphatases (probably PP2A) rapidly remove these phosphates when cyclin levels decline at the beginning of anaphase. Thus, the evidence supports the existence of a potentially important double-negative feedback loop in the control of APC/C activation. This is an important concept that will be of interest to experts in the field.

In general, the data support the authors' conclusions. I have the following concerns:

1. There is previous evidence that APC/C phosphorylation enhances its binding to Cdc20. It is clear from some of the experiments in this paper (Fig 5A) that APC/C phosphorylation does not seem to be sufficient for Cdc20 binding if the Cdc20 is phosphorylated. This seems reasonable. However, there are also some experiments earlier in the paper that seem to show that dephosphorylation of the APC/C does not reduce Cdc20 binding. Figure 2A and C, for example, show that the N159 fragment binds the APC/C very well despite dephosphorylation of Apc3. The authors should make some attempt to explain this potentially confusing result.

2. In Figure 1E, how is it possible that N159 phosphorylation inhibits its function in an extract that is later shown to contain a phosphatase that removes the phosphorylation?

3. The authors' results seem to indicate that the PP2A activity acting on the key sites in Cdc20 is constant through the cell cycle (Figure 3), although the data in this figure is rather inconclusive because we are looking at total phosphatase activity in extracts. In the discussion, however, the authors talk about the possibility that phosphatase activities increase in anaphase. In the end, I am not sure what we think about the identity and regulation of the phosphatases that target Cdc20. Some clarification would be helpful. Given that Cdc20 dephosphorylation coincides with cyclin destruction and Cdk inactivation, it is plausible that the phosphatase acting on Cdc20 is constant through mitosis.

4. The anti-phosphoT79 antibody is a very useful tool for some of the experiments, and the results with this antibody provide nice support for the authors' conclusions. However, the paper does not contain any characterization of the antibody. Such antibodies can be nonspecific or have other problems, and the authors should provide evidence that the antibody is specifically recognizing phosphorylated T79 in Cdc20 and not some other site on Cdc20 or any other protein.

5. The final experiments of the paper, shown in Figure 6, address the related but quite distinct question of the binding site on the APC/C for the Cdc20 N-terminal region. The authors show that a recombinant N-terminal region binds to Apc6 or Apc8 subunits expressed individually in insect cells. They also show that the C-box interacts with Apc8, and a mutation in the Apc8 TPR region abolishes this binding. These are potentially interesting and important results, but they are undeveloped and seem premature. As the authors mention in their discussion, previous evidence from yeast suggests that the C-box does not bind to Apc8 (Cdc23) but instead interacts with Apc2, and so the authors need to do more to resolve this discrepancy. It would be useful, for example, to provide some evidence that this interaction occurs in vivo with an intact APC/C and not just an individually expressed subunit. One possibility would be to remove these data and develop them into a full story for a separate paper. The current paper would then be focused entirely on the interesting question of Cdc20 regulation by phosphorylation.

6. In my opinion, it is time to retire the names 'Fizzy' and 'Fizzy-related', and use the generally accepted names 'Cdc20' and 'Cdh1'. Only a handful of people still use the Drosophila names, and general readers are likely to be confused by the use of these outdated terms.

1st Revision - authors' response 10 May 2012

RE: Manuscript EMBOJ-2012-80859

Point-by-Point response to reviewers' comments

Reviewer #1

1) I am concerned that the amino-terminus of Cdc20 may not recapitulate the behavior of the intact Cdc20 protein in all circumstances. The authors should replace the endogenous Cdc20 in cycling extracts (figure 5E) with a full-length Cdc20 phospho-mimetic (5E) or a full-length alanine mutant (5A) to compare the onset of Cyclin B and securin destruction. There is also a concern with Xenopus extracts as a model for mitosis since they are in essence a meiotic system. (It would greatly strengthen the paper if the authors showed that the de-phosphorylation of T64, T68, T79 is required for normal progression through mitosis by siRNA and rescue in human cells, for example.) In the absence of this the authors should include appropriate caveats in the text.

Nek2A is an excellent model substrate to monitor the APC/C activity in mitosis as well as meiosis and it binds the APC/C directly via the C-terminal MR motif. It can be degraded through mitosis (prometa-, meta- and anaphase) as long as the C-box in the N-terminal domain of Cdc20 is bound to the APC/C and activates the E3 ligase activity (Hayes et al., 2006; Kimata et al., 2008). In this manuscript, we wanted to focus on how the phosphorylation of the N-terminal domain affects the 'activation role' of Cdc20. Since phosphorylation possibly affects other properties of Cdc20 such as substrate recognition via the C-terminal WD40 domain, we deliberately used the N-terminal fragments of Cdc20 in conjunction with Nek2A as a substrate in order to strictly evaluate the phosphorylation effect on the activation role.

Having said so, we had included the full length Cdc20 in our manuscript (Figure 5A). The result clearly demonstrates that the full length Cdc20 is inhibited by phosphorylation, but non-phosphorylatable Cdc20-5A is free from such inhibition. Now we have also performed a new experiment using Cdc20 full length constructs in destruction assays similar to the one presented in Figure 1E using cyclin B and securin as substrates. The new data are presented in Supplemental Figure S3. From this result, the N-terminal fragments seem to recapitulate the behavior of the intact full length Cdc20, in terms of regulation of the activation domain. The new result also demonstrates that the phosphorylation effect is not specific to Nek2A and observed in the destruction of typical APC/C substrates such as cyclin B and securin.

Furthermore, as requested, we have replaced the endogenous Cdc20 in cycling extracts with a full length construct (Cdc20-FL) and an alanine mutant (Cdc20 5A-FL). The addition of either protein can induce two cycles in the depleted-extract, indicating that both Cdc20-FL and Cdc20 5A-FL can recapitulate Cdc20 functions. The kinetics of cyclin destruction are very similar, but this destruction is slightly accelerated with Cdc20 5A-FL, consistent with our model. The new data are presented in a new Figure 5E and Supplemental Figure S8.

Finally, we would like to make comments on the use of Xenopus extracts as a model system. It has been widely used to study many biological aspects including cell cycle control. CSF extract is meiotic metaphase II-arrested extract, but we can create mitotic extract (anaphase extract) by adding non-degradable cyclin B to interphase extracts. Moreover, we can make 'cycling extract' as well. In order to study the relationship between CDK phosphorylation and the 'activation role' of Cdc20, we have used CSF (meiotic) extracts, anaphase (mitotic) extracts and cycling extracts together and we believe that our data are solid.

2) The authors show that the phosphorylated N terminus of Cdc20 cannot support the degradation of Nek2a (figures 1E, 4C). How can this result be reconciled with the SAC-independent degradation of Nek2a in prometaphase when CDK activity is high and T64, T68, T79 are likely to be fully phosphorylated?

Our data suggest that Cdc20 phosphatases are active throughout mitosis, so we speculate that T64/68/79 are not fully phosphorylated in prometaphase, and thus a small amount of non-phosphorylated Cdc20 can activate the APC/C even when the SAC is active and degrade early mitotic substrates such as Nek2A and cyclin A. If Cdc20 is stably and fully phosphorylated by CDK in the presence of OA, it becomes inactive (See WT-p in Figure 4B, C, E and F). In Figure 1E, we did not combine OA treatment and pre-phosphorylation, so stable phosphorylation was not achieved, resulting in a pool of N159 free from inhibition as the phosphatases in the extract

continued dephosphorylating Cdc20.

3) The authors should quantify the assays shown in Figure 4B and 5A and present these as means +/- SDs.

As requested, we have quantified at least 3 independent experiments and presented the results with error bars (new Figure 4B, C, E and F and Figure 5A).

During this revision, we have found that CDK/cyclin A can use ATP more efficiently than ATPgS, particularly in the phosphorylation of T79 in the 2T constructs (T64/T79, T68/T79). Thus, we decided to repeat all the experiments (Figure 4B and 4C in the original manuscript) using ATP. But unlike thio-phosphorylation, phosphates can be removed by phosphatases, so we added OA to maintain phosphorylation during the assay. Please note that WT and 3T were both efficiently phosphorylated using either ATP or ATPgS. The inhibitory effect of thio-phosphorylated Cdc20 is clearly demonstrated in the New Supplemental Figure S3.

4) The authors should modify the Introduction. They make the surprising statement that structural data on APC/C is limited and fail to cite the recent structural papers from the Barford lab (Schreiber et al., 2011; da Fonsec et al., 2011). They also state that Cdc20-APC/C degrades securin and Cyclin B in anaphase whereas the key phase for this is metaphase. (Note that anaphase, as defined by sister chromatid separation, cannot take place if securin is not degraded.) Lastly, they should mention that Cdh1 is primarily regulated by Emi1 in interphase in animal cells.

As requested, the introduction has been modified.

Minor Points:
a) The authors should indicate molecular mass markers on all Western blots in figures 1-6

As requested, molecular mass markers are added on the Western blots.

b) Error bars should be added to the quantifications shown in figures 2B, 3A-C and 3F.

As requested, error bars have been added.

c) I don't think that the data in Fig 1E justify the statement that okadaic acid abolishes the activation by N159: reduces would be more appropriate.

As requested, we have changed the statement to that okadaic acid ‘reduced’ the activation.

d) The reference to Figure 6C needs to be added to the text (page 13)

As requested, we have added the reference to Figure 6C on page 14.

e) The model (figure 7) should be more precise regarding the mitotic phases (i.e. prometa-, meta-, anaphase) in which Cdc20 phosphorylation and de-phosphorylation occurs.

Unfortunately, our experimental system does not differentiate prometa-, meta- and anaphase. The main finding of this manuscript is that phosphatases are vital to activate the APC/C by dephosphorylating Cdc20, allowing its binding and activation of the APC/C. Our model presented in Figure 7 highlights this finding. To draw a more detailed regulation, further investigation will be clearly required, but it is beyond the scope of this manuscript.

Reviewer #2

1) To check how quickly the T79 phosphorylation of Fizzy is dephosphorylated in anaphase, the authors used anaphase egg extract supplemented with p27 (Figure 5C). But p27 forces the extract to

exit from anaphase and to go into interphase (judging from the downshift of Apc3 shown in the top panel). So this setting does not directly reflect the turnover speed of T79 phosphorylation in anaphase. It would be better to see the speed of the loss of radioactivity from N159-T79 fragment (by using Cdk and [g-32P]-ATP). If this is compared with the S50, S114 or some other stably-phosphorylated substrate, it would be perfect.

As requested, we have now measured the rate of loss of radioactivity from the N159-T79 fragment with reference to S50 and S114. T79 is clearly dephosphorylated with faster kinetics than S50 and S114. The experiment has been repeated 3 times independently and quantified. The data are presented in the new Supplemental Figure S7.

2) Figure 1B, 1C and S1A should have stained gel images, as the quantity of added proteins are important here.

As requested, added proteins resolved in SDS-PAGE and stained by CBB are presented in Supplemental Figure S1 and S2.

3) Page 7, lines 7-9. The description about Figure 1E is an exaggeration. The N159 fragment does support Nek2A degradation (lanes 9-16), if you compare it with lanes 1-4 of the same panel. Of course, I agree that OA and phosphorylation of N159 weaken (but not abolish) the impact of N159 on Nek2A degradation.

As requested, we have softened the expression to “reduces”, rather than “abolishes”.

4) Please describe in the main text why MG132 was added in the Figure 1A. Is this to avoid mitotic exit that could be induced by 5A protein?

CSF extracts were used for this experiment, so the APC/C is inactive due to XErp1 (an APC/C inhibitor). MG132 was added to ensure that cyclin destruction is blocked and the extracts stay in the CSF-arrested condition with high CDK kinase activity. The explanation has been added in the text (see page 8). However, as you can see in Figure 2A, regardless of the presence or absence of MG132, N159 can bind a similar amount of APC/C in both conditions. Please note that N159 cannot induce mitotic exit even using the 5A construct, because the N-terminal fragment (N159) cannot support destruction of cyclin B.

5) The position (mobility) of Apc3 in the right panel of Figure 2C looks intermediate, compared with that in the left panel. Is this phosphorylated (mitotic) form? Or is Apc3 partially dephosphorylated by the addition of 5A?

The positions of Apc3 in the right and left panels of Figure 2C are the same. The molecular weight markers in both Western blots substantiate this.

In fact, as shown in Figure 5D, N159 has a dominant negative effect on endogenous Cdc20 and thus when 5A is added, cyclin B destruction is blocked and high CDK activity is maintained, resulting in hyper-phosphorylation of Apc3.

6) Readers would need some explanation about the CSF state and differences between CSF (a kind of metaphase?) and anaphase extract.

As requested, we have mentioned this in the text (page 6 and 7).

7) What kind of egg extract was used in the Figure 2C? Probably it is CSF, but not clearly described anywhere.

It is CSF, and now it is clearly described in the Figure legend.

8) The symbol (-p) for normal phosphorylation in the Figure 1D & 1E is same as one for thiophosphorylation in the Figure 4B & C. Or is the N159 protein used in the Figure 1E also thiophosphorylated? If they are different, please use different symbols for clarity.

As requested, two different symbols for clarity, phosphorylation (-p) and thio-phosphorylation (-thio-p) have been used. However, as mentioned in reply to reviewer 1 (point 3), in this revised manuscript, we only used thio-phosphorylation for the new experiment presented in Supplemental Figure S3.

9) Please specify what IVT is, when it is first used (page 31, line 15).

Now, IVT has been replaced by 'in vitro-translated' throughout the text.

10) Please specify the delta-Cb mutation. Is this a substitution mutant or depletion?

This is a substitution mutant and the C-box (DRFIP) has been changed to AAAAA (Kimata et al., Mol. Cell 2008).

11) Boundaries of the short Fizzy fragments are not clear in the text or figures. For example, is 27th amino acid of Fizzy included in the fragment 1, 2 or both of them (Figure 1B)?

The 27th amino acid is included in fragment 1, but not fragment 2, since fragment 2 starts from the 28th amino acid. To clarity the information of the boundaries, we have listed all the peptides with amino acid numbers in the Experimental Procedures.

Reviewer #3

1) There is previous evidence that APC/C phosphorylation enhances its binding to Cdc20. It is clear from some of the experiments in this paper (Fig 5A) that APC/C phosphorylation does not seem to be sufficient for Cdc20 binding if the Cdc20 is phosphorylated. This seems reasonable. However, there are also some experiments earlier in the paper that seem to show that dephosphorylation of the APC/C does not reduce Cdc20 binding. Figure 2A and C, for example, show that the N159 fragment binds the APC/C very well despite dephosphorylation of Apc3. The authors should make some attempt to explain this potentially confusing result.

At the moment, we do not know which phosphorylation of APC/C subunits is responsible for Cdc20 binding, however, phosphorylation of Apc3 might not to be the key, in particular for the N-terminal Cdc20 binding. Therefore, in Figure 2A and C, although Apc3 is mostly dephosphorylated, Cdc20 efficiently binds the APC/C. However, it is still possible that phosphorylation of the APC/C (including phosphorylation of Apc3) is monitored by the C-terminus of Cdc20. It will be intriguing to identify the responsible phosphorylation sites within the APC/C and their regulation, however it is beyond the scope of this manuscript. We have added a brief explanation in the Discussion.

2) In Figure 1E, how is it possible that N159 phosphorylation inhibits its function in an extract that is later shown to contain a phosphatase that removes the phosphorylation?

N159 is highly phosphorylated by CDK2/cyclinA before adding to the extracts. The phosphorylation status of N159 during the assay is dependent upon the balance of phosphatases and kinases in the extracts. In this condition (Figure 1E), although phosphatases are present in the extract, most of N159 is phosphorylated at T64/68/79 and fails to support efficient destruction of Nek2A. However, some N159 is dephosphorylated and it can then support Nek2A destruction, so Nek2A degradation is slow, but not completely inhibited. If phosphorylation of N159 is maintained by blocking dephosphorylation, N159 becomes inactive (See WT-p in Figure 4B, C, E and F).

3) The authors' results seem to indicate that the PP2A activity acting on the key sites in Cdc20 is constant through the cell cycle (Figure 3), although the data in this figure is rather inconclusive because we are looking at total phosphatase activity in extracts. In the discussion, however, the authors talk about the possibility that phosphatase activities increase in anaphase. In the end, I am not sure what we think about the identity and regulation of the phosphatases that target Cdc20. Some clarification would be helpful. Given that Cdc20 dephosphorylation coincides with cyclin destruction and Cdk inactivation, it is plausible that the phosphatase acting on Cdc20 is constant through mitosis.

Yes, the reviewer is correct. Our phosphatase assay monitors total phosphatase activity, but it can monitor phosphate removal from a specific phosphorylation site on Cdc20. Our results demonstrate that T64/68/79 phosphatases are active and quite constant throughout mitosis. Also, we show that these are OA-sensitive phosphatases, but we suspect that more than one phosphatase is involved. They might all be constant throughout mitosis or one may be constant and the other activated at a specific time, such at the metaphase to anaphase transition when APC/C^{Cdc20} becomes active. In addition, in Xenopus egg extracts, there is no SAC activity unless it is treated with microtubule depolymerizing agents such as nocodazole and high concentrations of sperm nuclei. In this manuscript, we did not investigate the relationship between T64/68/79 phosphatases and the SAC. However, as we have discussed in the Discussion, it is possible that the SAC might regulate the phosphorylation status of Cdc20 by regulating Cdc20 kinase(s) or Cdc20 phosphatase(s). We believe that such a detailed analysis is beyond the scope of this manuscript.

4) The anti-phosphoT79 antibody is a very useful tool for some of the experiments, and the results with this antibody provide nice support for the authors' conclusions. However, the paper does not contain any characterization of the antibody. Such antibodies can be nonspecific or have other problems, and the authors should provide evidence that the antibody is specifically recognizing phosphorylated T79 in Cdc20 and not some other site on Cdc20 or any other protein.

The anti-phosphoT79 antibody (BT2.1) is specific to phosphorylated T79. Only T79 phosphorylated peptide could block BT2.1, but unphosphorylated peptide could not. In addition, in our Western blot, the single mutation on T79 to A (T79A) abolishes the BT2.1 signal, although the protein has four other CDK sites (S50, T64, T68 and S114). The characterization of the antibody is now presented in the new Supplemental Figure S6.

5) The final experiments of the paper, shown in Figure 6, address the related but quite distinct question of the binding site on the APC/C for the Cdc20 N-terminal region. The authors show that a recombinant N-terminal region binds to Apc6 or Apc8 subunits expressed individually in insect cells. They also show that the C-box interacts with Apc8, and a mutation in the Apc8 TPR region abolishes this binding. These are potentially interesting and important results, but they are undeveloped and seem premature. As the authors mention in their discussion, previous evidence from yeast suggests that the C-box does not bind to Apc8 (Cdc23) but instead interacts with Apc2, and so the authors need to do more to resolve this discrepancy. It would be useful, for example, to provide some evidence that this interaction occurs in vivo with an intact APC/C and not just an individually expressed subunit. One possibility would be to remove these data and develop them into a full story for a separate paper. The current paper would then be focused entirely on the interesting question of Cdc20 regulation by phosphorylation.

We do not think that we have discrepancy with previous results as they used Cdh1, not Cdc20. Regulation of Cdh1 and Cdc20 are not necessarily the same. In fact, Cdh1 activates the APC/C regardless of the phosphorylation status of the APC/C, whereas Cdc20 only activates the APC/C when it is phosphorylated. Cdh1 and Cdc20 might interact with different APC/C subunits via the N-terminal domain, as we speculate in the Discussion. Also, both previous experiments are from budding yeast APC/C, not vertebrate APC/C. Furthermore, the previous result (Thornton et al., 2006) did suggest that Cdh1 interacts with Apc2, but did not show that the interaction is dependent upon the C-box. Thornton et al also mentioned in the Discussion that deletion of Apc2 might affect the ability of Cdc27/Apc3 or other subunits to bind Cdh1. In this manuscript, we have shown that the N-terminal of Cdc20 binds Apc8 in a C-box-dependent manner. As far as I know, this is the first evidence that the C-box interacts with an APC/C subunit specifically. Although the interaction is not very strong, it is clearly significant and C-box dependent. Since Cdc20 (and Cdh1) binds the APC/C via the C-terminal IR motif and the N-terminal domain and both are required for efficient binding, it is very difficult to measure the physical interaction in vivo. We have found that mutating either domain drops the binding to the APC/C to 5~10%. A recent report from the Pines laboratory clearly suggests that Cdc20 binds the Apc8 subunit and activates the APC/C in mitosis.

Recent elegant work from the Barford laboratory has uncovered the cryo-EM structure of the APC/C, however, the structural information of Cdh1 lacked the N-terminal domain including the C-box. Thus, it is possible that Cdh1 binds Apc2 via another motif within the N-terminal domain, other than the C-box.

In the Discussion, we have included a couple of possibilities to explain the difference between Cdc20 and Cdh1, but the detailed analysis of the mechanism of loading of Cdc20 and Cdh1 onto the APC/C is beyond the scope of this manuscript.

6) In my opinion, it is time to retire the names 'Fizzy' and 'Fizzy-related', and use the generally accepted names 'Cdc20' and 'Cdh1'. Only a handful of people still use the Drosophila names, and general readers are likely to be confused by the use of these outdated terms.

We try to respect the history and people who discovered and firstly described these genes in Drosophila, however, as a reviewer suggested, should it confuse general readers, we are happy to retire the names, Fizzy and Fizzy-related. Now, as requested, we have changed to ‘Cdc20’ and ‘Cdh1’.

2nd Editorial Decision 24 May 2012

Thank you for submitting your revised manuscript for our consideration. It has now been seen once more by two of the original referees (see comments below), and I am happy to inform you that there are no further objections towards publication in The EMBO Journal. The only minor point retained by referee 1 is the introduction of a brief caveat (most appropriately at some point in the discussion) regarding pure mitotic vs meiotic/mitotic extract systems. Therefore, I would appreciate if you simply sent us a new text file containing such a short addition at your earliest convenience. We would then replace the version in our manuscript tracking system, and after that should be able to swiftly proceed with formal acceptance and production of the manuscript!

Yours sincerely

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1

The authors have addressed all of my criticisms with one exception. I asked that the authors insert a caveat that the Xenopus system may not entirely recapitulate a mitotic system and the authors argued against this. There are, however, data from Thomas Mayer, amongst others, that Xenopus extracts retain a number of meiotic proteins, such as Xerp1/Emi2 that alter the characteristics of the extracts. Thus, I would like to ask again that the authors insert a caveat that their findings may not be directly applicable to a purely mitotic system.

Referee #3

The authors have performed a wide range of new experiments and text revisions. These changes have addressed my previous concerns, and in my opinion the paper is eminently suitable for publication.

Revision received 25 May 2012

Please find attached new text file.

To satisfy referee 1, in the caveat, I used the expression "plausible" in the previous version, but I think that "conceivable" is a better expression. So, I would like to change as follows (page 17 in the text).

However, it is also conceivable that our findings in Xenopus egg extracts may not be fully applicable to somatic and/or mitotic cells, because cell-free extracts prepared from unfertilised

Xenopus eggs retain a number of meiotic proteins and lack some of the key somatic proteins such as Cdh1.

If you have any questions or concerns, please do not hesitate to contact me.

